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# AMINO ACID INCORPORATION INTO PROTEIN BY RABBIT SPLEEN AND LYMPHNODE MICROSOMES

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Incorporation of labeled amino acids into proteins was studied in the spleen and lymph nodes of normal and immune rabbits. The pH 5 fraction from spleen and lymph nodes resulted in very low incorporation. The pH 5 fraction from rabbit liver was therefore used. The properties of the protein synthesized were studied. Immunological methods failed to demonstrate the presence of γ-globulin.

Now data are available showing that cell-free systems of bacterial and ani-/516* mal origin can incorporate amino acids into definite proteins normally synthesized by cells which are used to obtain microsomes or ribosomes (refs. 1 and 2). For example, the microsomal fraction of the rat's liver is capable of incorporating amino acids into proteins which are similar in immunological properties to albumin and to a series of other proteins synthesized by the liver (refs. 3 and 4).

An effort has been made in a series of laboratories to demonstrate the incorporation of amino acids into γ-globulins or antibodies in a cell-free system of lymphatic tissues (refs. 5-8). In two cases successful results were described (refs. 7 and 8). Subsequently, however neither the authors nor other investigators confirmed these preliminary reports.

The purpose of the present work has been the study of the inclusion of tracer amino acids into the proteins of a microsomal fraction which was obtained from the spleen or the liver of immunized and normal rabbits. Certain properties of the radioactive protein were also investigated.

## Materials and Methods

### Immunization

The investigations used rabbits which were immunized with serum albumin of man and globulin from a bull. The rabbits were sacrificed on the fourth day, after repeated immunization. Usually one immune rabbit yielded 5-7 g of spleen and inguinal, popliteal, and axillary lymph nodes.

### Microsomes

The tissues were finely minced with scissors and immersed 10 min into a hypotonic solution (0.01 M tris buffer, pH 7.4; 0.015 M $MgCl_2$; 0.01 M KCl and

*Numbers given in margin indicate pagination in original foreign text.

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0.006 M mercaptoethanol). Then the gentle mixture was transferred to a homogenizer and the cells were destroyed by 2-3 gentle movements of Teflon pestle. The homogenate was subjected to centrifugation for 20 min at 17,000 g and the microsomes were precipitated from the supernatant liquid for a period of 60 min at 105,000 g. The microsome pellet was carefully weighed into a solution containing 0.035 M tris buffer, pH 7.8; 0.01 M $MgCl_2$; 0.065 M KCl and 0.006 M mercaptoethanol (incubation medium).

The pH 5 fraction was used as a source of the necessary enzymes and sRNA. The tissue was homogenized in 2.5 volumes of the incubation medium with 0.15 M sucrose and then all particles were precipitated successively at 17,000 g and 105,000 g (60 min). Before precipitation of the pH 5 fraction the supernatant liquid, after centrifugation at 105,000 g, was diluted twice with water and 1 N acetic acid was used to reach a pH of 5.2-5.1. The precipitate was separated by centrifugation at 12,000 rpm in the cold, washed once with water and dissolved in the incubation medium specified above.

Incubation was carried out with continuous stirring in a water bath at 36.5° in conical flasks. In the incubation medium there were 3-5 mg of microsome protein and 4-7 mg protein of the pH 5 fraction per ml of suspension. In addition to this 10 micromoles of potassium phosphoenolpyruvate, 2 micromoles of ATP, 0.3 micromoles GTP, 100 mg of phosphoenolpyruvate-kinase, .0 millicurie of Chlorella $C^{14}$-protein hydrolysate and 0.006 M equimolar mixture of 19 amino acids or a solution of methionine, serine, tryptophan and threonine. /517

Specimens.

Phosphoenolpyruvate was synthesized by the method described by Ohlmeyer (ref. 9). The final yield of silver-barium salt of phosphoenolpyruvate was 2 grams from 15 grams of pyruvic acid. The silver-barium salt was converted to a potassium salt by solution in 0.2 N HCl and the precipitation of barium with 0.5 M $K_2SO_4$. The precipitate was separated by centrifugation and the supernatant liquid was made alkaline with 1 N KOH.

The ATP (manufactured in Hungary) was applied in the form of an alkaline solution of disodium salt. GTP was transformed from the barium salt into the potassium salt by treating it with KU-2 resin.

Phosphoenolpyruvate kinase was isolated from rabbit muscle using the method described by Beiseherz and others (ref. 10); 900 mg of singly recrystallized enzyme were isolated from 2.9 kg of muscle.

Protein was determined by the Lauri method: the radioactivity of the protein was determined by the Mans and Novelli method (ref. 11) with minor changes. A Whatman 3MM paper disk 23 mm in diameter was wetted repeatedly with the test solution and dried with a stream of hot air for 20-30 sec. Following this the disk was immersed in cooled 10-percent TCA. If necessary, the disk was again covered with the solution with periodic drying. On the next day the disks were processed in a manner similar to the classical method of Sekivits. The radioactivity was measured in a liquid scintillation

counter. In each case 3-4 duplicate disks were used. Judging from specially conducted repetitive experiments this method of counting radioactivity is 17 times more efficient than the method using an end-window counter. (the same quantity of pure tracer radioactive antibodies, synthesized in the tissue, was compared in ref. 12).

Results and Discussion

Investigation of the capability of spleen and lymph node microsomes of a rabbit to incorporate amino acids into protein. The method of disintegrating cells used by us yielded 5-6 mg of microsomal fraction protein from one gram of wet weight of rabbit spleen and lymph nodes. In this case we did not find a clear difference between normal and immunized animals.

Initially a study was made of the capability to incorporate amino acid proteins in the presence of the pH 5 fraction isolated both from the spleen and lymph node supernatant as well as from the rabbit liver supernatant. It turned out that in the first case the incorporation is more than ten times lower (62 and 850 counts/min/mg microsomes respectively). Apparently this is due to the presence of a large quantity of RNAse and proteolytic enzymes in the lymphoid tissue. For the same reason, apparently, the very low incorporation into the protein was observed in the homogenate of lymph nodes and spleen (250 counts/min/mg of microsome). Therefore, in subsequent experiments we used only the pH 5 fraction from the rabbit liver.

The incorporation of tracer amino acids into the protein by microsomes of the lymphatic tissue varies from experiment to experiment between 1000 and 1500 counts/min/mg of microsomes. No noticeable differences were found between inclusion into protein by microsomes from normal and immunized rabbits. These values are approximately 1.5-2 times greater than what has been obtained by Wust and Novelli (ref. 13) for incorporation into the ribosome protein in a cell-free system of rat spleen with the addition of pH 5 fraction of rat liver. The incorporation of the tracer into the protein by microsomes from rat liver in our experiments was 2.5-3 times greater than by microsomes from the lymphatic tissue of a rabbit. This in one of the experiments inclusion constituted 3400 counts/min/mg protein of microsomes from rat liver.

In a series of experiments we studied the transition of tracer proteins into the solution and investigated some of their properties. If, after incubation, the particles are precipitated by centrifugation at 105,000 g for a period of one hour, it turns out that the supernatant contains only a small percentage of the total radioactive protein--approximately 15 percent. However if deoxcholate is added to the suspension to achieve a final concentration of 0.5 percent and RNAse at a concentration of 30-50 mg/ml, and if then the suspension is passed through a column with Sephadex G-25 in 0.005 tris buffer solution, pH 7.8 with 0.0001 M $MgCl_2$, and if the particles are

precipitated only after this, then the supernatant liquid acquires a substantially higher percentage of the tracer protein. For example, in one such experiment in which the inclusion of microsomes into the protein was 1460 counts/min/mg protein, the following results were obtained (see table). /518

Thus approximately 50 percent of the tracer protein passed into the supernatant liquid. We can presume that this was not associated with the destruction of the ribosomes themselves by RNAse because at the levels of enzyme used the intactness of the ribosomes is preserved (ref. 14). The figures which have been obtained are approximately of the same order as those in the experiments of Hultin on the solubility of tracer protein from the liver ribosomes (ref. 15).

| | Radioactivity of the protein counts/min/0.1 ml |
|---|---|
| Suspension before incubation | 756 |
| Suspension after incubation | 3686 |
| Suspension after addition of deoxycholate, RNAse and passage through Sephadex G-25 | 2534 |
| Supernatant liquid after centrifugation at 105,000 g | 1296 |
| Precipitate after centrifugation at 105,000 g | 1378 |

Investigation of the tracer protein from the supernatant. First of all we determined the approximate size of the protein molecules containing the radioactive tracer. For this purpose the supernatant fraction after treatment with deoxycholate and RNAse was passed through columns of Sephadex G-50 and G-75. The quantity of protein and radioactivity were measured in the fractions which resulted. It turned out that the activity per mg of protein in both cases is the same before and after gel filtration. Apparently radioactivity is associated with protein molecules of large size.

In subsequent experiments the supernatant liquid was subjected to fractionation using the Sephadex G-200 gel (ref. 16). The column (15 x 400 mm) was fed with 3 ml of the supernatant containing 2.9 mg/ml of protein with an activity of 600 counts/min/mg of protein. The results of this experiment are shown in the figure. We investigated the activity of high-molecular-weight fractions which immediately left the column and three fractions which were held back by the gel. It turned out that in fraction 12 the protein had an activity of 490 counts/min/mg, while in fraction 22 it had an activity of 392 counts/min/mg of protein. Thus radioactive protein is contained not only in the first fractions where we can expect large fragments of ribosomes, but also in the subsequent fractions which contain protein held back by the gel.

In other experiments we checked to see whether the radioactivity was associated with the small peptides bound into larger units by noncovalent bonds. For this purpose urea was added to the supernatant liquid up to 6M and formic acid up to 0.05 M, and the solution thus obtained was passed through a column of Sephadex G-25 in 6M urea and 0.05 M formic acid, i.e., under conditions when the noncovalent bonds are broken (ref. 17). It turned out that if the activity before passage was 714 counts/min/mg of protein, then after passage through the column the activity was 543 counts/min/mg of protein. Thus there was no substantial retardation of the radioactive protein by the gel. This points to the fact that in acidic urea the principal part of the radioactive protein retains its size.

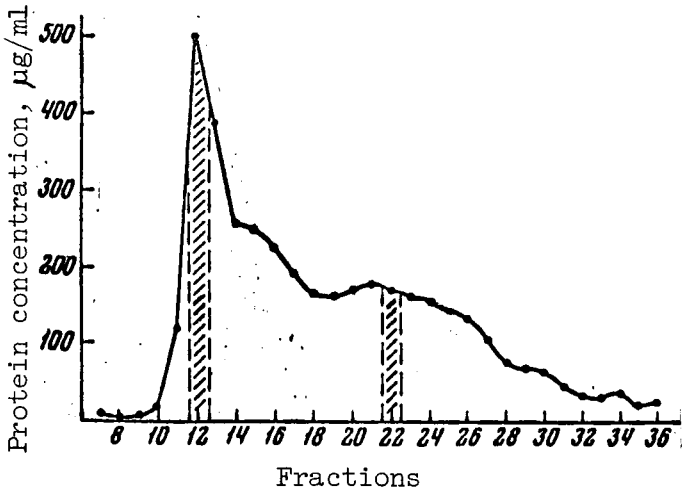


Figure. Fractionation on the Sephadex G-200 gel of soluble proteins in the incubated suspension of rabbit spleen and lymph-node microsomes. The shaded areas indicate fractions whose radioactivity was investigated.

Finally in a series of experiments with immune rabbits we determined the capacity of the radioactive protein of the supernatant liquid to react with various types of immunosorbents. Clear positive results were not obtained in experiments with antigens fixed on cellulose: reliable increase in radioactivity was not established on such sorbents. In other experiments we checked to /519 see whether there is any radioactive protein in the supernatent which would be bound by the complex of rabbit γ-globulin (RGG) fixed on cellulose and the antibodies of an ass against RGG. As shown by Drizlikh and Gurevich (ref. 18) such sorbents are capable of binding the corresponding antigen from the solution. Indeed in our experiments 0.2 ml (2.0 mg) of RGG fixed on the cellulose which has been processed with serum against RGG bonded approximately 40 micrograms of RGG from the solution. However in no case did such a sorbent bind the radioactivity from the supernatant liquid. Negative results also occurred in the effort to bind by means of the sorbent-antibody the radioactivity which existed in the supernatant after centrifugation of microsomes treated with ultrasonics (22 kHz, 3 times for 30 sec at 0°). Although ultrasonic irradiation of this type removed the tracer albumin from the microsomes of rat liver (refs. 3 and 4) the detection of protein with the solubility properties of RGG was not possible.

Thus in spite of the fact that the microsomes of rabbit spleen and lymph nodes are capable of incorporating amino acids into proteins, it was not possible for us to show that, under our conditions, the tracer is incorporated into the antibodies or the γ-globulin. It is possible that the liver pH 5 fraction is not entirely suitable for this purpose. Another rather probable reason is that when the cells are destroyed the liberated RNAse destroys the integrity of the polyribosomes which usually participate in the formation of polypeptide chains. This possibility in particular is indicated in the experiments of Scharff and Uhr (ref. 19) who showed that extracts of lymphocytes destroy the polyribosomes of the HeLa cells.

## CONCLUSIONS

The incorporation of $C^{14}$ amino acids into proteins is accomplished by the microsomal fraction of immune and normal rabbit spleen and lymphocytes only in the presence of the pH 5 fraction of rabbit liver. The same fraction of lymphoid tissue was almost inactive. On the average the incorporation per mg of microsomal protein is 2.5-3 times lower compared with that observed with rat liver microsomal proteins.

Processing of the incubated microsomal suspension with deoxycholate and RNAse, and subsequent gel filtration through Sephadex G-25 at low ionic strength resulted in the liberation of approximately half of the tracer protein into the supernatant fraction. During fractionation on Sephadex G-200 the tracer turned out to be associated with the proteins which did not penetrate the Sephadex G-200 granules as well as with proteins retained by the gel. The radioactive protein did not bind to antigen fixed on cellulose nor to the antibody sorbent (fixed rabbit γ-globulin treated with serum against this antigen). Consequently no protein possessing antigenic activity of γ-globulin is synthesized under the above conditions. /520

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